THE EMBRYOLOGY OF THE SPLEEN IN *AMIA CALVA*

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INTRODUCTION

INVESTIGATIONS on the spleen of higher vertebrates have failed to clarify many of the problems related to its embryology, to the vascularization of its pulp, to the significance and the potentialities of its first cellular components, to its relations to the reticulo-endothelial system and other lymphoid organs, to its relations to iron, calcium, fat, lipoid, and antibody metabolism, and to the effect on the animal of its removal. Many investigators have turned to the study of the spleen in lower forms in the hope of obtaining information that might aid in the correct interpretation of the organ in higher forms. In the present investigation an approach to some of these problems has been made through a study of the morphogenesis and the histogenesis of the organ in one of our fishes, *Amia calva*. The differentiation of its cell types has been followed, and an attempt has been made to interpret the findings on a functional basis.

The observations of Piper (1902*a*, 1902*b*, 1903) on the development of the spleen in *Amia* were incidental to his study of the development of the other visceral organs, and those of Robeson (1932) dealt only with hemopoiesis in the adult. Among the more recent and important papers on the spleen in other fishes are those of Jordan (1924, 1933) and of Jordan and Speidel (1924*a*, 1930, 1931), which deal with the evolution of the organ and its relation to blood-cell formation. Its histology has been described by Maximow (1923) in *Acanthias*, by Hemmeter (1926) in *Alopecias vulpes*, and by Scatizzi (1932) in *Chimaera monstrosa*, and a comparative study of several forms was made by Yoffey (1929). De Gaetani (1926) described the arterial terminations in the pulp of the spleen in *Scylliorhinus canicula*. The development of the organ was first described by

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Laguesse (1890) in *Acanthias* and *Trutta fario*. His findings have recently been confirmed by Léon (1932), who followed the history of the cell types by the use of vital stains. Numerous papers dealing with the spleen in other forms have appeared, but they will be referred to only so far as they have some bearing on the development of the organ in *Amia*. Good reviews of the literature on the spleen are given by Theil and Downey (1921), Fowler (1924), Krumbhaar (1926), Von Skramlik (1927), and others.

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MATERIALS AND METHODS

The spleen and related structures were studied in approximately 170 individuals, varying in age from the time of hatching (6.5 mm. body length) to maturity. The sequence of the series up to 20 mm. varied by a millimeter or less, and those above 20 mm. by not more than 5 mm. Correlation of the different phases of splenic development with early embryonic processes proved to be impossible because of the advanced stage at which the splenic primordium makes its first appearance. Consequently total body length was chosen as an indication of age, admittedly a poor criterion, but sufficiently accurate for the purposes of this investigation.

The macroscopic structure of the spleen was studied directly from dissections and from geometrical reconstructions of serial sections. The blood vessels were injected with color masses and India ink. Injected specimens were fixed in Carnoy's II and cleared *in toto* with synthetic oil of wintergreen.

The microscopic structure was studied from section and smear preparations. The tissues were fixed in Kolmer's, Carnoy's II, Helly's, Zenker's, and Wittmaak's fluids. Most of the sections were cut at 5 microns and stained with iron hematoxylin and eosin, Mallory's triple, Wright's, or Giemsa's azure-eosin. From a group of half-grown to adult fish the following types of smear preparations of the splenic pulp were made: (1) fresh smears of cells suspended in Ringer's solution; (2) smears, fixed by drying or by formalin vapors and stained with Wright's or Giemsa's azure-eosin; (3) spleen-blood serum smears (Isaacs, 1928) made by macerating

the splenic pulp in blood serum, mounting the macerate, and then fixing and staining as in 2; (4) anatomical smears made by spreading the residue from thoroughly macerated spleen-blood serum mixtures on a slide, drying it just enough so that it would stick, and then treating the preparation as in 2. Excellent preparations of the reticulum were obtained by this method. The resulting distorted shape of the cells is an obvious disadvantage of this method, but this is compensated for by the fact that the cells, with their long cytoplasmic processes, are isolated from the pulp cells, so that the reticulum cells may be studied in their entirety instead of in fragments, as in serial sections.

MORPHOGENESIS

In this investigation Piper's (1902*a*, 1902*b*, 1903) and Robeson's (1932) observations have been confirmed in the main, but elaborated in detail and reinterpreted in the light of more recent research. For convenience of description it has seemed desirable to refer to the various stages of splenic development as "the long mesenchyme bed," the "club-shaped spleen," and the "hooked spleen."

The Long Mesenchyme Bed

The splenic primordium appears first in larvae of about 7.5 mm. body length as an elongated band of mesenchyme following the course taken by the subintestinal vein. The anterior end lies on the left side of the intestine, and it spirals ventrally so that its posterior end lies on the right side of the gut. The main axis of the primordium makes an angle of about 40° with the mid-sagittal body plane. Before the larvae are 10 mm. in body length, the exact limits of the splenic area are rather indefinite; however, the primordium becomes conspicuous by a condensation of its mesenchyme and an accompanying increase in the mitotic divisions of its cells. The splenic primordium lacks an arterial supply at this early age (Pl. LXII, Fig. 1), and its vascularization is entirely venous in character. The original subintestinal vein has differentiated into the anterior (dorsal) and the posterior (ventral) intestinal veins and the newly developed splenic vein joins the latter along the posterior third of the splenic primordium. No secondary branches leading from the splenic primordium are noticeable at this age.

The Club-Shaped Spleen

In larvae between 12 and 13 mm. body length there occur a more pronounced condensation and a localization of the splenic primordium. This change is characterized by a marked increase in the number of cells in the spleen. The general development, however, takes on a different aspect. In the earlier stages there was a crowding of the increasing number of mesenchyme cells into a more limited space, but now the spleen is beginning to change its shape by localized growth, finally resulting in a reduction in the total length of the organ. The change in the shape of the splenic primordium from a long mesenchyme bed to a shorter, thicker, and more condensed form (Pl. LXII, Fig. 2) suggests the "club-shaped" spleen of Piper's descriptions, and I have adopted this designation. The two ends are heavier than the central region, and the anterior end is slightly larger than the posterior. As a result of the bending of the intestine, the organ becomes relatively straight. In position it is almost parallel to the axis of the intestinal spiral and the mid-sagittal plane of the body; however, its anterior end lies at a more ventral level (about 40°) than its posterior end. The organ as a whole has begun to separate from the intestinal wall, and it may now be considered an independent constituent of the viscera. During the progress of this separation an omental connection is retained along the whole length of the organ.

At this age all the fundaments for the vascularization of the spleen are definitely formed, but the actual blood supply of the organ is still principally venous in character. The splenic vein receives a few secondary branches from within the organ, but the splenic artery, which has differentiated and lies parallel to the vein, is without secondary vessels penetrating the organ (Pl. LXII, Fig. 2). The intestinal vessels of this region lie in the omentum, and in some cases they appear to follow the organ during its separation from the intestine, but this relationship is only a temporary one.

The Hooked Spleen

In larvae 13.5 mm. in length there is an approach to conditions as they are found in the adult. The general shape of the spleen may be described as "hooked" (Pl. LXII, Fig. 3). The bending of the intestines to form the first complete loop and the accompanying

torsion or twisting have brought the hooked spleen from a position ventral to the intestinal tract, to a position just dorsal to the junction of the large and small intestines. Its main axis, which was formerly more or less parallel to the axis of the intestinal spiral, is now plainly perpendicular to the latter. The spleen has also shifted in its relations to the mid-sagittal plane, and the anterior end now lies to the right. The anterior half of the organ is predominantly larger; its tip is bending to the left and ventrally. The posterior half remains slender, and its tip also bends ventrally, thus maintaining its original relationship to the inner curvature of the spiraling intestine. The omental support of the hooked spleen is narrowing down and is attached to only the anterior and central regions of the spleen.

The major blood vessels of the splenic region lie in the intestinosplenic omentum. The posterior intestinal arteries and veins approach the central region of the spleen, but the splenic artery and vein are very closely associated with the posterior lobe of the spleen. The splenic artery may have two to four secondary branches penetrating the splenic tissue, and the vein may receive as many as fifteen secondary branches from the spleen.

In the 14.5 mm. larva the position and the shape of the spleen are the same as in the adult. Figure 4 (Pl. LXII) represents these definitive conditions. With continued looping of the intestine the anterior end of the spleen is carried to the left again, so that its major axis now lies almost parallel to the mid-sagittal body plane. The organ as a whole lies just anterior and dorsal to, and is closely attached to, the junction of the large and small intestines. This position is retained throughout the life of the animal. The anterior end of the organ has grown considerably both toward the right and ventrally. It constitutes the anterior lobe and has not been observed to subdivide further into lobules. With its increase in size it becomes plainly visible in a ventral view of the visceral organs, where it lies just anterior to the intestinal junction, and posterior and ventral to the posterior tip of the cardiac stomach. Similarly, the posterior tip of the spleen, or posterior lobe, is visible ventrally, even though it lies back of the interintestinal omentum and within the posterior intestinal curvature. The central sector, or middle lobe, is characterized by a hilum along its left ventral edge. It retains its close relations to the intestinal junction by the attachment of the intestinosplenic omentum, which is confined to the hilum.

At this stage (Pl. LXII, Fig. 4) the original splenic artery and vein join the posterior intestinal artery and vein at right angles at the hilum and have become incorporated within the organ. Secondary branches increase in number by constant subdivision, but no characteristic plan of arrangement can be recognized among them.

Discussion of Morphogenesis

The position of the spleen in the adult *Amia* must be considered extraenteric as described by Jordan (1924). This is similar to the conditions found in elasmobranchs, other ganoids, and teleosts. During development there is a constant change in position with increasing age. The long mesenchyme bed and the club-shaped spleen lie ventral to the intestinal tract, but the hooked spleen lies dorsal to it. These changes are not the result of the movements or of the migration of the spleen itself, but of the twisting and looping of the intestinal tract. The position of the long mesenchyme bed is determined by the course of the subintestinal vein, while the positions of the club-shaped and the hooked spleens are determined by the attachment of the intestinosplenic omentum and its relations with other visceral organs. The anterior lobe of the hooked spleen, as in the adult, is crowded ventrally by the posterior tip of the stomach. More posteriorly the intestinal loop forces the posterior lobe of the spleen into the small space between the large and the small intestines just posterior to their junction, and consequently its chance for free growth is limited.

The vascularization of the spleen described for each of its phases of development must be interpreted in the light of conditions as they are found in the adult. In general, the vessels may be designated as forming the extrinsic and the intrinsic systems. The former includes the vessels that are found near the hilum and outside the organ, namely, the posterior intestinal arteries and veins (Pl. LXII, Fig. 4), and these veins include those which are incorporated within the organ and enter at the hilum, namely, the splenic arteries and veins with their primary, secondary, and tertiary branches. The development of the arteries is not synchronized with the development of the veins in the early stages. The details of this process will be discussed later.

HISTOGENESIS OF THE SPLEEN IN *AMIA CALVA*

The Origin of the First Cellular Components

The first cells that contribute to the formation of the splenic primordium may easily be traced back to the mesenchyme cells along the outer wall of the subintestinal vein. However, in larvae between 6 and 7.5 mm. body length the mesenchyme has not yet invaded this area (Pl. LXIII, Fig. 1). At this stage the coelomic epithelium consists of a fairly distinct single layer of cells. The nuclei of its cells are round to oval, and slightly larger than those of the mesenchyme cells. The peri-intestinal mesenchyme is a homogeneous loose-celled tissue clearly set off from the intestinal epithelium by a distinct basement membrane. The nuclei of the tissue are round to oval in section and may each contain one or two nucleoli; the cytoplasm is slightly granular, and cell membranes are indistinguishable. The beginnings of the subintestinal vein may be recognized by its differentiating endothelium, which is clearly defined only on the proximal surface of the vessel. On the distal surface the endothelium is incomplete, and there is free communication between the lumina of the differentiating vein and that of the venous yolk plexus. The young blood cells in the vessel have presumably been derived from the blood islands on the surface of the yolk mass.

In larvae between 8 and 9 mm. in length the peri-intestinal mesenchyme, from which the splenic primordium originates, has just begun to differentiate in the area between the coelomic epithelium and the endothelium which forms the distal wall of the vein (Pl. LXIII, Fig. 2). The nuclei of these cells are smaller and less conspicuous than those of the neighboring mesenchyme cells, and their cytoplasm is relatively clear and completely fills the interspaces. Irregular masses of the cells may project into the lumen of the vein in the regions where the endothelium is still incomplete. The coelomic epithelium is now a distinct layer, and forms a complete covering for the early splenic primordium.

The Long Mesenchyme Bed

The coelomic epithelium, the primordium of the capsule, changes very little during this stage, except that the nuclei seem to project more prominently into the coelomic cavity, giving a pebbled effect to the surface of the organ (Pl. LXIII, Fig. 3). The mesenchyme

cells multiply actively by mitosis to form the pulp, the most conspicuous part of the organ. The great majority of its cells have very lightly stained and inconspicuous nuclei. These seem to be differentiating into the reticular tissue of the pulp. A few other cells, having large, oval, slightly reticulated nuclei with a moderate amount of basophilic cytoplasm, apparently are young stages of the erythrocyte series or lymphomyeloid hemoblasts, as described by Robeson (1932) in the adult *Amia*.

At this time small irregular clear spaces begin to appear between the mesenchyme cells. These tissue spaces or lacunae increase in size by a pushing back of the neighboring cells. The cells on their surface tend to flatten and form a pseudoendothelial lining. Several adjacent lacunae have been observed to fuse into larger lacunar spaces. Occasionally a hemoblast migrates into the lacunar space and immediately matures into an erythrocyte.

The endothelial lining of the subintestinal vein remains essentially as in earlier stages. Occasional hemoblasts from the splenic pulp enter the lumen of the vessel along its distal wall, where the endothelium is still incomplete.

The Club-Shaped Spleen

This stage in splenic histogenesis (Pl. LXIII, Fig. 4) is characterized by a continuation of lacunar differentiation in the pulp, by fusion of the lacunae to form more extensive sinusoidal spaces, and by the fusion of some of these spaces with the lumen of the splenic vein. This fusion occurs in the regions where the distal endothelial wall is not completed by a true endothelial lining and is a significant phase in splenic development because it results in the establishment of a communication between the deeper parts of the splenic pulp and the general venous circulation. The splenic artery, which has differentiated, lies in the peri-intestinal mesenchyme, but has no branches extending into the pulp (Pl. LXIII, Fig. 4). It contains blood cells, and its endothelial lining is very distinct. It differentiates by an apical growth of its tip, which penetrates between the mesenchyme cells.

In this stage a new type of cells is differentiating from a few of the mesenchyme cells of the pulp. They are considerably larger than other cells, their cytoplasm contains acidophilic granules, and their nuclei are spherical and have a lightly reticulated chromatin

network. They resemble the early stage of an eosinophil as described by Robeson (1932).

Another change which is begun at this age is the deposition of isolated masses of dark brown granular pigment in the splenic pulp. Each of these masses is composed of from 30 to 50 granules. These, however, are not peculiar to the splenic tissue, but appear in the body wall and other organs as well.

The Hooked Spleen

In the development of the splenic capsule its outer component, the single layer of mesothelial cells derived from and continuous with the peritoneum, remains almost unchanged. It covers the entire organ except at the hilum. Growth of the organ, however, has resulted in a stretching of these cells, so that they form a layer only 4 to 6 microns in thickness, with nuclei appearing spindle-shaped in sections. In larvae of 16 to 20 mm. body length there is a differentiation of the mesenchyme cells beneath the visceral mesothelium to form the second component of the capsule, namely, the collagenous connective tissue layer. Its fibrils take on a bluish color in Mallory's triple connective tissue stain. Their differentiation continues until the larvae are about 50 mm. in length, when the adult condition seems to be established. The whole fibrous layer may vary in thickness from 6 to 10 microns. It is difficult to make out the limit of its inner surface, since the underlying reticulum is attached to and is continuous with it. The reticulum in these peripheral regions of the pulp is comparatively coarse, and when studied in sections shows some clear spaces. These probably belong to a system of subcapsular lymph channels. No muscle cells have been observed in any stage of capsular development.

The differentiation of the pulp of the hooked spleen into the two major regions described by Robeson (1932) in the adult spleen, namely, the white pulp or lymphoid cords which follow the course of the main blood vessels, and the red pulp or myeloid tissue which fills in all the interspaces, does not become apparent until the fish is almost half grown. In the earlier stages, 14.5 mm. larvae, only the red pulp or myeloid tissue exists. It consists of a basic framework of mesenchymatous cells, which retain their embryonic characteristics until the larvae are about 17 to 20 mm. long. Then those cells which are destined to become part of the permanent framework

of the pulp differentiate. This change can be detected in the appearance of collagenous fibrils in their cytoplasm, similar to those described for the capsule, but in the 25 mm. larvae it is found throughout the pulp as in the adult (Pl. LXIV, Fig. 1). It should be noted that this true reticulum is the supporting framework for the pulp, and its component strands of tissue make a veritable meshwork or net, in the meshes of which the free elements of the pulp are lodged. There is no evidence of the trabecular development which is characteristic of the spleens of higher vertebrates. The undifferentiated mesenchyme cells which retain their embryonic characteristics have been interpreted by various investigators (Laguesse, 1890; Jordan and Speidel, 1930; and others) as stem or mother cells of the future hemoblasts and especially of the lymphocytes.

Multiplication of the hemoblasts by mitosis and their differentiation into all types of blood cells continues as in the earlier stages. Erythropoiesis proceeds at the normal rate, and the great increase in the number of mature erythrocytes in the interstices of the reticulum can be accounted for only by the establishment of an arterial supply to the splenic pulp (to be described later) and an influx of these cells from the general circulation. Leucopoiesis, or the formation of granulocytes, as they are named by Robeson (1932), continues in part in the pulp. The eosinophils especially are more numerous and conspicuous in the lumina and walls of the veins, the peri-intestinal mesenchyme, and the omentum. Evidence seems to indicate that their progenitors are the large cells found in the lumen of the splenic vein of the club-shaped stage. The special granulocytes, which originate from the granuloblasts, tend to remain in groups of three to six in the pulp. Many contain ingested erythrocytes and pigment granules indicating phagocytic activity. Basophils are also present. They are very heavily granulated and may also contain remnants of the red blood cells. Eosinophils with bacillary granules, monocytes, and thrombocytes, described by Robeson (1932) from the blood stream of the adult, could not be identified in the developing splenic tissue.

The masses of brown pigment, which first appeared in the club-shaped spleen, form a third component of the red pulp. These masses increase in number and size, and may at this stage contain 50 to 100 granules each. Their relations to any of the splenic cells remain obscure and no satisfactory explanation of their origin could

be obtained. They resemble closely the pigment found in other parts of the body.

The lymphoid regions of the pulp do not become apparent until the young fish is at least 150 mm. long. Then great numbers of both the large and the small lymphocytes appear as isolated nodules near some of the smaller blood vessels. These masses grow and fuse with one another, so that in the adult spleen about half of the pulp is lymphoid in character. According to Robeson (1932), the small lymphocytes are derived from the larger type. Jordan (1933) contends that in teleosts they may differentiate into other types of blood cells after entering the blood stream and that both types originate from the hemoblasts. This contention holds for the young *Amia* as well. In sections of the spleen the lymphocytes are very abundant, but the number of cells in mitosis is very small; consequently local division can scarcely account for their great numbers.

During the development of the blood vessels in the hooked spleen the veins remain quite independent of the arteries, although the major intrinsic vessels, the splenic artery and vein, lie parallel to each other in the hilar region. In the pulp no constant plan of arrangement has been recognized among their branches.

The early development of the intrinsic venous trunks has been traced to the sinusoids of the club-shaped spleen. In the hooked spleen the structure of the vessels characteristic of the adult is soon established. The larger venous trunks, in the spleens of larvae 15 to 18 mm. long, acquire a definite endothelial lining which differentiates directly from the flattened and exposed mesenchyme cells. At the same time some of the underlying mesenchyme cells differentiate into a thin layer of supporting connective tissue with collagenous fibrils such as has been described for the capsule. In the half-grown fish a thin layer of circular, smooth muscle fibers appears between the endothelium and the fibrous connective tissue layer to complete the structure of the larger intrinsic veins. The terminations of the veins in the pulp resemble the embryonic conditions in that the endothelium gradually blends into the reticulum and the lumina remain in direct communication with the open spaces or the venous sinuses of the pulp (Pl. LXIV, Fig. 2). Openings, or stomata, of this type also occur in the sides of some of the smaller venules.

The important histogenic feature of the hooked spleen is the establishment of an arterial supply to the pulp. In the 13.5 mm.

larvae the first two branches of the splenic artery penetrate the pulp from the outside and enter at the hilum. They appear to have a growing tip which forces aside the mesenchyme and free elements of the pulp. It is interesting to note that an erythrocyte usually lies within the tip of the growing point and probably aids in expanding the lumen. The mesenchyme cells along its path differentiate to form the fibrous connective tissue layer of the vessel. At the hilum the fibrous layer is continuous with the connective tissue of the main extrinsic artery and the splenic capsule. It has been called the hilar sheath by Whiting (1897). Collagenous fibrils, which become continuous with those of the reticulum, appear in its cells in the 17 mm. larvae. In the adult the fibrous layer is ten to twenty times as thick as the corresponding layer of the veins. In the half-grown fish circular and longitudinal muscle fibers appear in the walls of the larger arteries, but their development was not followed.

The terminations of the intrinsic splenic arteries in the pulp are different from the veins because they develop as a closed system of tubes; hence some means other than the usual capillary system is devised to complete the vascular circuit. Soon after the vessels have penetrated the pulp, in 13.5 to 20 mm. larvae, they branch and rebranch irregularly to form arterioles. These arterioles lack the muscular layer, and their lumina are about 15 to 20 micra in diameter. In the 25 to 50 mm. larvae two to four thick masses of the mesenchyme cells radiating from the ends of the arterioles tend to become clear of all cytoplasmic granules, and their nuclei stain lightly. The blood cells are pushed aside, since a small branch of the arteriole grows into each of these regions. The arterial ends are ellipsoids (Pl. LXIV, Fig. 3). Within the ellipsoids the endothelium blends with the reticulum and loses its characteristics as a lining membrane. The lumina remain very narrow, and the distal ends come into direct communication with the open sinusoid spaces of the pulp. Later disintegrating erythrocytes and isolated pigment granules are frequently found in the walls of the ellipsoids. Yoffey (1929) and Scatizzi (1932) both claim to have found deposits of lipoid granules in the ellipsoids. Figure 3 (Pl. LXIV), taken from the spleen of the adult, shows a branch of an arteriole and the major part of three ellipsoids. In the lower ellipsoid the open communication with the pulp spaces is apparent. In the upper ellipsoid a degenerating erythrocyte can be seen. The final result is the estab-

lishment of an open vascular circuit through the splenic pulp and an influx of blood cells, especially erythrocytes, from the general circulation into the open meshes of the reticulum.

Discussion of Histogenesis

There has been some difference of opinion regarding which germ layer gives rise to the spleen, and the controversy has centered principally around the spleen of ganoids. There are those who believe in an entodermal origin, whereas others maintain that the origin is entirely mesodermal. The literature on this subject has been summarized by Thiel and Downey (1921). My observations on the development of the spleen in *Amia* lead me to agree with those who maintain that the organ is of mesodermal origin, and they confirm especially the conclusions reached by Piper (1902*a*, 1902*b*, 1903), who worked on the same form. The splenic primordium in *Amia* takes its origin from the mesenchyme along the outer wall of the subintestinal vein. Piper referred to this fundament as the long mesenchyme bed. This terminology I have adopted. Léon (1932) holds that the vascular endothelia give rise to splenic mesenchyme. In *Amia* the evidence indicates that there is a migration of the perintestinal mesenchyme into the potential splenic region and that this is followed by a rapid proliferation of the mesenchyme cells, some of which differentiate into endothelial cells, quite in contrast to the view held by Léon.

With regard to the splenic capsule, my observations indicate that its two main components, the mesothelium and the underlying collagenous fibrous connective tissue, each arise from different sources. The former is continuous with and derived from the coelomic epithelium. It remains as a thin single-layered epithelium and in the adult appears as a smooth glistening peritoneal covering in every way similar to that of the other visceral organs. The fibrous layer is a derivative of the mesenchyme of the pulp and develops considerably later (18 mm. larvae). Laguesse (1903) made an extensive study of the development of the capsule in *Acanthias*, and came to the conclusion that the collagenous fibrils of the capsule originated as a hyalinization and a solidification of some precollagenous material in the mesenchyme cells. A study of sections of the spleen of *Amia*, which were stained with Mallory's triple connective tissue stain, indicates that the conclusion reached by Laguesse is correct. This

transformation, which takes place gradually, begins in certain peripheral or subcapsular regions of the organ and around the larger blood vessels and then spreads slowly through the entire pulp. In successively older stages the affinity for the aniline blue increases both in intensity of the stain and in amount of material stained. This process occurs simultaneously with the penetration of the arteries into the pulp, but it is doubtful whether there is any physiologic relation between the two processes, since the arteries are closed tubes at this time and the blood does not have a free circulation through the pulp.

Whiting (1897) called the thick layers of connective tissue surrounding the main intrinsic arteries an involuted hilar sheath. He believed that this sheath was derived directly from the capsule of the organ, and he placed considerable emphasis upon this interpretation. My study of this layer in *Amia* indicates that it develops *in situ* and that there is no involution of the capsule to form it. It is continuous with the capsule, but it is also continuous with the homologous layer in the extrinsic part of the artery. However, Whiting was correct in his suggestion that the major blood vessels do offer some support for the pulp through their relations with the reticulum.

The major regions of the splenic pulp in fishes are generally described as the white or lymphoid regions and the red pulp or Billroth cords. Yoffey (1929), working on the spleen of elasmobranchs, describes three regions, namely, the lymphoid, the pulp, and the ellipsoids. Most earlier workers considered the ellipsoids a part of the arterial system. Hemmeter (1926), in his study of *Alopecias vulpes*, speaks of them as hemolytic islands. In the adult *Amia* the ellipsoids are directly connected with the ends of the arterioles; however, in development they are derived from the mesenchyme cells and acquire their relations to the arteries secondarily. Yoffey's interpretation of them as separate regions in the splenic tissue is correct, but of little significance.

A study of the cell types found in the splenic pulp involves a careful analysis of questions relating to hemopoiesis, cell lineage, hemolysis, pigment formation, and differentiation of mesenchyme cells. It is beyond the scope of this investigation to consider blood-cell formation and destruction in detail, but a few general observations have been included in the light of Robeson's description (1932)

of hemopoiesis in the adult *Amia*. Most of his interpretations, so far as they involve the spleen, have been substantiated.

An interesting observation has grown out of the comparison of the erythroblasts found in the ordinary anatomical smear preparations with those found in spleen-blood serum smear preparations. In the latter the erythroblasts respond by maturing into erythrocytes and also by entering into mitosis. There apparently is some stimulus in the latter treatment which calls forth these responses. Jordan and Speidel (1924*b*) concluded from their studies on intravascular maturation of the erythroblasts that the necessary stimulus was contact with the blood stream and a lower carbon dioxide concentration. It is possible that similar conditions may be operative in the spleen-serum technique.

An analysis of the development of the early mesenchymal cells indicates that these must be interpreted as the polyvalent parent cells of the various elements of the splenic pulp. Evidence for this has been presented, and it has been shown that they may differentiate into endothelial, reticular, hemoblast, and ellipsoidal cells and that they may also produce pigment. The parent cells do not display any characteristics by which their subsequent fate might be determined. They divide actively to produce more cells of the same kind even in the adult spleen or they may differentiate at any time into the definitive types. This view is also supported by Jordan in several recent publications, and he goes a step further by contending that the lymphocytes are the mother cells for all types of blood cells.

I can add little to the question of the origin of splenic pigmentation. General observation indicates that there are two types of pigment. The isolated dark brown granular masses first observed in the club-shaped spleen may be secretion products of certain cells of the splenic pulp. The other type of pigment is found in the cells of the ellipsoids and in certain granulocytes. Krumbhaar (1926), Yoffey (1929), Scatizzi (1933), and others have presented clinical and experimental evidence which indicates that these granules may be disintegration products of hemolysis of the senile erythrocytes. In *Amia* these granules and decomposing red blood cells are found in the granulocytes and in the ellipsoids at the same time.

The development of the blood vessels supplying the spleen in *Amia* indicates that there is a close functional relationship between

the organ and the vascular system. The vessels enter the hilum and branch extensively to supply the pulp, where they form an "open" circulation through the meshes of the reticulum. Thiel and Downey (1921) have given an accurate summary of the conflicting opinions regarding the terminations of the finer vessels within the spleen of various vertebrates. Modern investigators such as Krumbhaar (1926), De Gaetani (1926), Tait (1927), Yoffey (1929), McNee (1931), and Scatizzi (1932) all have come to the conclusion that an "open" circulation exists through the splenic pulp in fishes, a conclusion to which the earlier workers were not reconciled.

The intrinsic splenic veins begin their development about the same time that the splenic primordium first appears. Their differentiation proceeds from within the splenic pulp, and involves the formation of lacunar spaces and their fusion to form sinusoids. These processes take place in the long mesenchyme bed, but the union of the larger sinusoids with the lumen of the subintestinal vein does not take place before the stage of the club-shaped spleen. The differentiation of the mesenchyme cells into the endothelial and the connective tissue layers of the larger vessels is not completed before the stage designated as the "hooked spleen." Their differentiation is similar to that described by Laguesse (1890) in his study of the developing spleen of *Trutta fario* and recently confirmed by Léon (1932).

That the arteries penetrate the organ from the outside is an important embryological feature and quite in contrast to the development of the veins which grow from within the pulp toward the hilum. Similar observations were made by Tait (1927) in *Acanthias*. It is also of importance that the arteries begin as a closed system of tubes, which later acquire open terminations by way of the ellipsoids. It has not been possible to discover how the penetration is effected, but the possibility may be suggested that the growing tip of the artery may have some digestive action on the tissues penetrated, or perhaps the penetration results from a pressure on the inside of the arterial ends. It is also significant that the arterial supply of the spleen is established much later in the development than the venous supply. The fact that the splenic artery remains relatively small as compared with the vein is of importance. It emphasizes the venous origin of the spleen, and the arrangement allows for a lower blood pressure, and a slower current in the sinusoidal

spaces of the pulp, and also provides for a convenient storage place for all types of blood cells as well as an excellent locus for hemopoiesis and hemolysis. The strategic position of the ellipsoids with their hemolytic and phagocytic powers is of significance in connection with the elimination of undesirable substances that might enter the splenic pulp from the general circulation.

SUMMARY

1. The critical stages in the morphogenesis of the spleen of *Amia calva* have been described as the long mesenchyme bed, the club-shaped spleen, and the hooked spleen.

2. The splenic primordium originates in about the 7.5 mm. larva from the peri-intestinal mesenchyme along the outer wall of the subintestinal vein. During morphogenesis, changes in its position result from the twisting and looping of the intestinal tract. In the adult the organ occupies an extraenteric position, and is supported by the intestinosplenic omentum from the junction of the large and the small intestines.

3. The extrinsic and the intrinsic vascularizations of the spleen have been described. The veins of the splenic pulp are formed early in life through the formation of lacunae, the fusion of adjacent lacunae to form sinusoids, and the subsequent fusion of the sinusoids with the lumen of the splenic vein. The endothelial, muscular, and connective tissue cells which compose the walls of the veins are derived from the mesenchyme cells of the pulp. In the hooked spleen the arteries penetrate the pulp at the hilum as closed branches from the splenic artery, and they open into the pulp through the ellipsoids secondarily. An "open" circulation in the pulp has been demonstrated. The ellipsoids are derived from the mesenchyme cells of the splenic pulp and function as valves controlling the arterial supply to the pulp and also as phagocytic centers.

4. The major regions represented in the splenic pulp are the myeloid and the lymphoid. The histogenesis of the mesenchyme cells has been traced into the reticulum, endothelium, hemoblasts, and ellipsoids, and they probably give rise to pigment. In the hooked spleen the reticulum composes the major supporting framework of the pulp, in the interstices of which are many free cells, such as hemoblasts, erythroblasts, granuloblasts, erythrocytes, granulocytes, and pigment granules.

5. Throughout life the spleen is active in hemopoiesis and hemolysis, and as a storage place for all types of blood cells.

6. The capsule has a double origin from the coelomic epithelium and the mesenchyme. No muscle fibers have been observed in it, but these are present in the walls of the larger blood vessels. There is no evidence of trabecular development.

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ABBREVIATIONS USED IN PLATES

A.I.A. — Anterior intestinal artery
A.I.V. — Anterior intestinal vein
Duo. — Duodenum
H.P.V. — Hepatic portal vein
Int. — Intestine
L.Int. — Large intestine
Mes.V. — Mesenteric vessels
Pan. — Pancreas
P.Gas.A. — Posterior gastric artery
P.Gas.V. — Posterior gastric vein
P.I.A. — Posterior intestinal artery
P.I.A.D. — Posterior intestinal artery, dorsal
P.I.A.V. — Posterior intestinal artery, ventral
P.I.V. — Posterior intestinal vein
P.I.V.D. — Posterior intestinal vein, dorsal
P.I.V.V. — Posterior intestinal vein, ventral
P.M.A. — Posterior mesenteric artery
S.Int. — Small intestine
S.I.V. — Subintestinal vein
Sp. — Spleen
Sp.(A.L.) — Spleen (anterior lobe)
Sp.(M.L.) — Spleen (middle lobe)
Sp.(P.L.) — Spleen (posterior lobe)
Sp.A. — Splenic artery
Sp.V. — Splenic vein

EXPLANATION OF PLATE LXII

Figures 1, 2, 3, and 4 have been drawn from geometrical reconstructions of parts of the viscera of *Amia* larvae at the body lengths indicated. The arrows above and below each figure indicate the mid-sagittal body plane. All represent the dorsal view. The mesenteries and the omenta have been omitted for purposes of clarity. Part of the intestinal tract has been omitted to show the underlying splenic primordium.

Fig. 1. The long mesenchyme bed of an 11 mm. larva

Fig. 2. The club-shaped spleen of a 12.5 mm. larva

Fig. 3. The hooked spleen of a 13.5 mm. larva

Fig. 4. The hooked spleen of a 50 mm. larva. This closely approximates the adult position, the shape, and the vascularization of the organ

PLATE LXII

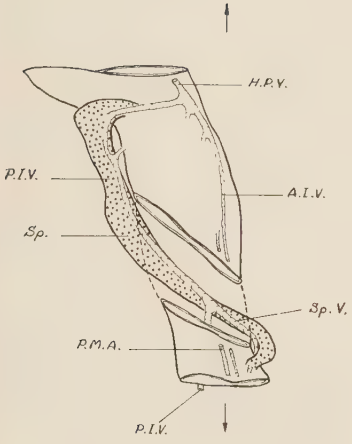

FIG. 1

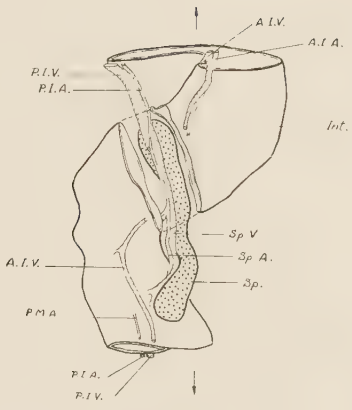

FIG. 2

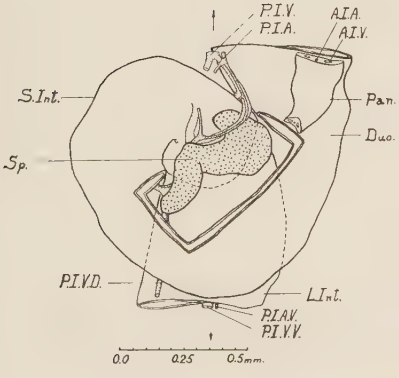

FIG. 3

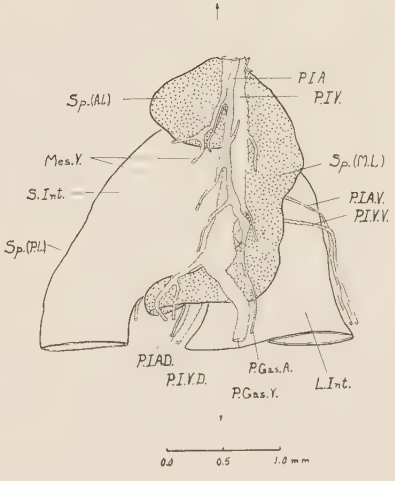

FIG. 4

EXPLANATION OF PLATE LXIII

FIG. 1. Photomicrograph of a cross section through the intestine of a 6.5 mm. larva. The peri-intestinal mesenchyme has not yet invaded the region where the splenic primordium will develop, and the lining of the subintestinal vein is not yet a distinct membrane

FIG. 2. Photomicrograph showing invasion of peri-intestinal mesenchyme between coelomic epithelium and subintestinal vein from which the splenic primordium will arise. 8 mm. larva

FIG. 3. Photomicrograph of cross section through the long mesenchyme bed. 10.7 mm. larva, showing fusion of the lacunae and some hemoblasts

FIG. 4. Photomicrograph of a cross section through the club-shaped spleen. 12.7 mm. larva

PLATE LXIII

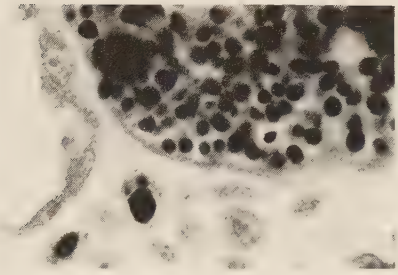

FIG. 1

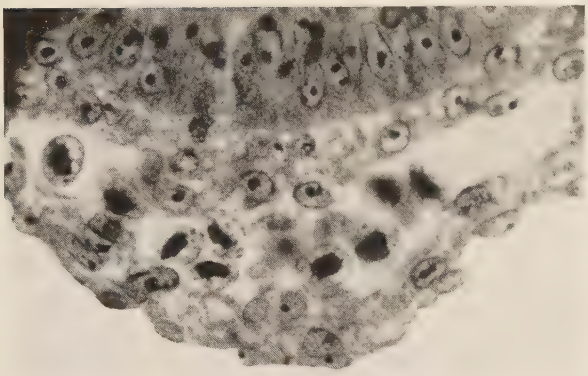

FIG. 2

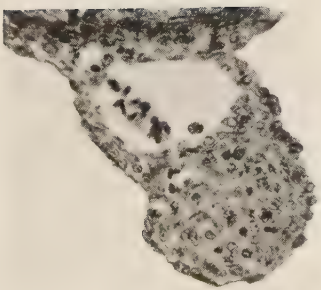

FIG. 3

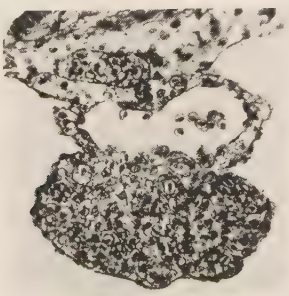

FIG. 4

EXPLANATION OF PLATE LXIV

FIG. 1. Photomicrograph of the reticulum of the pulp prepared from the residue of spleen-blood serum smears

FIG. 2. Photomicrograph showing a venule with its stomata. This reveals the manner in which free communication is established between the open sinuses of the pulp and the venous trunks. Taken from half-grown fish (24 cm.)

FIG. 3. Photomicrograph of a section through an arteriole and three of its ellipsoids. Taken from the adult spleen. Note the disintegrating erythrocyte in the upper ellipsoid and the open communication between the lumen of the lower ellipsoid with the sinuses of the pulp

PLATE LXIV

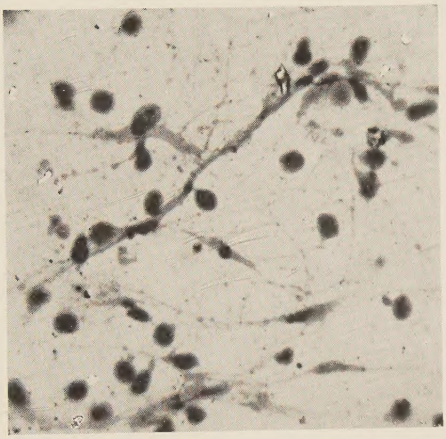

FIG. 1

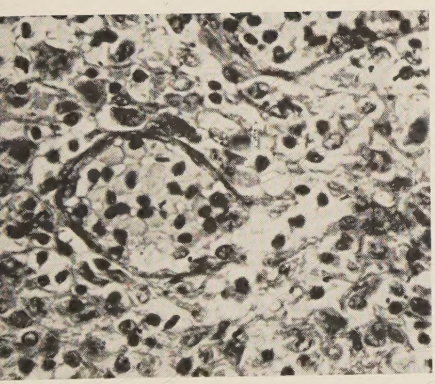

FIG. 2

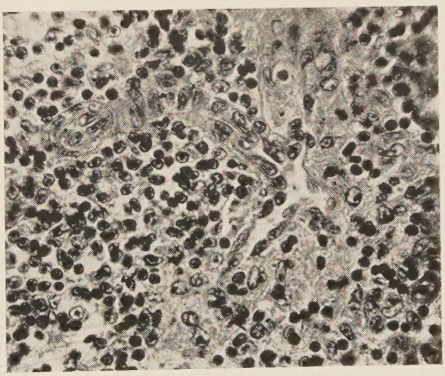

FIG. 3